

A method for identifying *Mycobacterium tuberculosis* and Mycobacteria other than Tuberculosis, together with detecting resistance to an antituberculosis drug of Mycobacteria obtained by mutation of *rpoB* gene

BACKGROUND OF THE INVENTION

This application claims priority to the foreign application KR 2001-43450 filed July 19, 2001.

Field of the Invention

The present invention relates to a method for identifying *Mycobacterium tuberculosis* (hereinafter, referred to as '*M. tuberculosis*') and Mycobacterium Other Than Tuberculosis (hereinafter, referred to as 'MOTT'), and for the determination of resistance of *M. tuberculosis* to an antituberculosis drug obtained by the mutation of the *rpoB* gene.

Description of the Related Arts

Tuberculosis is a chronic wasting disease caused by *M. tuberculosis*. Worldwide, it ranks the first in mortality and morbidity among infectious diseases (Global Tuberculosis Programme. Global Tuberculosis Control, WHO Report 1997. World Health Organization, 1997). Carriers of *M. tuberculosis* presently number about 1.9 billion, a third of the world population, and about 8-10 million of these carriers develop into new tuberculosis patients per year, and about 3 million of patients die of tuberculosis per year (Dolin P. J., Raviglion M. C., Kochi, A. (1994) Global tuberculosis incidence and mortality during 1990-2000. Bull World Health Organization. 72(2): 213-220; Kochi, A. (1992) The global tuberculosis situation and the new control strategy of the World Health

Organization. *Tubercle*. SCI. 72:1-6; Styblo K. Epidemiology of Tuberculosis the Hague, Royal Netherland Tuberculosis Association (1991), p. 83 in: Minister of Health and Welfare, The Korean National Tuberculosis Association. Measures for Tuberculosis Control in 2000s, p5, 1997). Also, about a half of the population in Korea are carriers of *M. tuberculosis*, and about 150,000 persons develop into new tuberculosis patients per year, and about 14,000 patients per year die of tuberculosis (Sreevatsan S. Stockbauer KE, Pan X, Kreiswirth BN, Mogha SL, Jacobs WR Jr, Telenti A, Musser JM. (1997) Ethambutol resistance in *M. tuberculosis*: critical role of *embB* mutations. *Antimicrob Agents Chemother* 41:1677-1681).

Recently, it was reported that patients doubly infected with HIV virus and *M. tuberculosis* are much more susceptible to tuberculosis. The seriousness of the problem of tuberculosis has become increasingly apparent, together with the increase of HIV in the world as well. Presently, it is estimated that about 15 million patients are double infected by both the *M. tuberculosis* and HIV. Also, most of these patients will probably develop into fulminant tuberculosis patients, which will lead to their deaths (Narain J. P., Ravigliione M. C., Kochi A. (1992) HIV-associated tuberculosis in developing countries: epidemiology and strategies for prevention. *Tuber Lung Dis*. SCI. 73(6):311-321).

The insufficiency of the present antituberculosis drugs and improper treatment and control of tuberculosis in these developing countries results in the increase of patients with *M. tuberculosis* which are the resistant to antituberculosis drugs (Global Tuberculosis Programme. Anti-tuberculosis drug resistance, WHO Report 1997. World Health Organization, 1997). The increasing appearance of resistant *M. tuberculosis* has resulted in the concomitant increase in tuberculosis related mortality and has obstructed attempts to eliminate it from a population (Ariel, P. M., M. C. Raiglion, A. Laszlo, N. Binkin, H. L. Rieder, F. Buster, D. L. Cohn, C. S. B. L. van weezenbeck, S. J. Kim, P.

Chaulet, P. Nunn. (1998) Global surveillance for antituberculosis-drug resistance. *New England J. of Medicine*. SCI. 338:1641-1649; Centers for Disease Control. (1992) National action plan to combat multidrug-resistant tuberculosis. *Morb Mortal Wkly Rep* 41(RR-11):5-48; Global Tuberculosis Programme. Global project on Anti-tuberculosis Drug Resistance Surveillance. WHO Report 1997. World Health Organization, 1997; Tuberculosis: A Global Emergency (news). *World Health Forum*, 14(4):438, 1993. In: Minister of Health and Welfare, The Korean National Tuberculosis Association. Measures for Tuberculosis Control in 2000s, 1997). In the meantime, current treatment of resistant *M. tuberculosis* is quite costly and has low efficiency, thus the resistant mycobacteria often develops into incurable tuberculosis. Therefore, earlier diagnosis of resistant *M. tuberculosis* should result in more efficient treatment of tuberculosis patients.

In most countries, including Korea, the drug-susceptibility test for *M. tuberculosis* is performed using standard microbiological methods, which require long time periods of 8-10 weeks to grow *M. tuberculosis* in culture (Index of Korean Health and Welfare in 1996, p. 412, Korea Institute for Health and Social Affairs, 1997). Therefore, methods for rapidly and accurately detecting the drug susceptibility of the infecting *M. tuberculosis* are needed. Rapid diagnosis of resistant mycobacteria may increase the efficiency of the tuberculosis treatment because it may provide proper treatment strategies earlier in the development of the disease. Ultimately, rapid diagnostic methods may slow the increase of incurable tuberculosis resulting from infection with drug-resistant *M. tuberculosis*. Therefore, such diagnostic methods are important health care technologies capable of decreasing further economic loss by prevention, elimination and treatment of tuberculosis.

Recently, the mechanism of drug-resistance was discovered by the use of genetic-based technology. For example, it is reported that the mechanism by which *M.*

tuberculosis obtains resistance to rifampin, one of the strongest effective antituberculosis drugs, results from a nucleotide mutation in the 69bp region of the gene which encodes the β -subunit of RNA polymerase (*rpoB* gene) (Telenti A., Imboden P., Marchesi F., Lowrie D., Cole S., Colston M. J., Matter L., Schopfer K., Bodmer T. (1993) Detection of rifampicin-resistance mutation in *Mycobacterium tuberculosis*. *Lancet*. SCI. 341:647-50; Vareldzis B. P., Grosset J., de Kantor I., Crofton J., Laszlo A., Felten M., Raviglione M. C., Kochi A. (1994) Drug-resistant Tuberculosis: Laboratory Issues, World Health Organization Recommendations. *Tuber Lung Dis*. SCI. 75(1):1-7). At least 97% of *M. tuberculosis* with resistance to rifampin is due to the above mutation.

The genus *Mycobacterium* includes, in addition to *M. tuberculosis*, *M. leprae* (Hansen's disease or Leprosy) and other *Mycobacterium* species generically called *Mycobacterium Other Than Tuberculosis* (hereinafter, referred to as 'MOTT'). MOTTs are mycobacteria which cause opportunistic infections, and thus MOTTs generally infect patients who have reduced immunity; however, normal persons can be infected on occasion. Since 1980, most of the industrialized nations have reported that MOTTs cause tuberculosis in HIV patients. Further, the number of diseases found to be caused by MOTTs has been increasing, so that the rapid and accurate identification of mycobacterial species has been recognized as being important for health care providers.

Conventional methods for identifying *M. tuberculosis* and MOTTs are based on various microbiological and biochemical properties of *Mycobacterium* species (Kochi A., Vareldzis B., Styblo K. (1993) Multidrug-resistant tuberculosis and its control. *Res Microbiol*. SCI. 144(2):104-110). However, such conventional identifications require for up to four weeks of growth time, depending on the types of mycobacteria, thus the identification time is prolonged. In addition, the results obtained by the conventional methods are sometimes unclear, and some *Mycobacterium* species cannot be

distinguished from other species with these conventional methods. Therefore, to overcome the above problems of the conventional identifications, the identification of MOTTs using molecular biological methods has been recently developed.

Particularly, methods for the molecular biological identification of MOTTs using as its target, a highly conserved genetic domain in all mycobacterial species, were developed (Timpe A, Runyon EH: The relationship of "atypical" acid-fast bacteria to human disease: A preliminary report. *J Lab Clin Med.* 44: 202, 1954; Jenkins, P. A.: Lipid analysis for the identification of mycobacteria. *An appraisal. Rev. Infect. Dis.* 3: 382-866, 1981; Tsang, A., I. Drupa, M. Goldgerg, J. McClatchy, and P. Brennan. 1983. Use of serology and thin-layer chromatography for the assembly of an authenticated collection of serovars within the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex. *Int. J. Syst. Bacteriol.*; Butler, W. R., K. C. Jost, Jr., and J. O. Kilburn. 1991. Identification of mycobacteria by high-performance liquid chromatography. *J. Clin. Microbiol.* 29:2468-2472, 33:285-292).

Among these, the *rpoB* gene found by the present inventors may be used in identifying MOTTs more simply and rapidly than the conventional molecular biological method using identification of the nucleotide polymorphism of 16S rRNA (KR99-46795).

That is to say, the *rpoB* gene includes highly conserved regions capable of being detected in all the species of mycobacteria. More importantly, the *rpoB* genes from Enterobacteriaceae are not amplified by PCR using the primers based on the *rpoB* gene sequence of mycobacterial species, or if amplified, the PCR products are distinguishable from each other. Therefore, the mycobacterial *rpoB* gene may be used in preparation of PCR primers specific to *Mycobacterium* species.

Further, the results of the above prior studies by the present inventors show that the *rpoB* gene of *Mycobacterium* includes nucleotide regions having polymorphisms.

Therefore, the *rpoB* gene may also be used in preparation of Mycobacterium species-specific probes for DNA-hybridization.

Conclusively, the present inventors found in prior studies that a 361bp region in the *rpoB* gene can be successfully used in the specific identification of *M. tuberculosis* and MOTTs.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method for specifically identifying *M. tuberculosis* and MOTTs, and to detect whether said mycobacteria has a mutation of the *rpoB* gene, rendering it drug resistant.

Another object of the present invention is to provide primers for PCR amplification of the *rpoB* gene used in identifying *M. tuberculosis* and MOTTs, and to detect the presence of the mutation of the *rpoB* gene.

Still another object of the present invention is to provide oligomer probes and a membrane to adhere said probes for performing reverse blot hybridization.

Yet another object of the present invention is to provide a kit for identifying *M. tuberculosis* and MOTTs, together with detecting the antimicrobial resistance of a mycobacterial species by detection of mutations of the *rpoB* gene, using oligomer probes adhered to the membrane and said primers, or which includes said primers, said oligomer probes and said membranes.

To achieve the above purposes, the present invention identifies *M. tuberculosis* and MOTTs, and detects whether the Mycobacterium has a mutation in the *rpoB* gene which would confer antibiotic resistance by the following method:

- (1) isolating DNA from a sample;
- (2) amplifying a 531bp fragment of the *rpoB* gene of the Mycobacterium by PCR

using said mycobacterial DNA isolated in step (1) as template, and using the primers MOTT-rpo-long-B-5' (5'-TCAAGGAGAAGCGCTACGACCTGGC-3'; SEQ. ID. NO. 1) and TR8-long-NB-3' (5'-ACGGGTGCACGTCGCGGACCTCCA-3'; SEQ. ID. NO. 2); and,

(3) performing PCR-reverse blot hybridization by hybridizing the PCR products obtained in step (2) to a membrane capable of adhering the oligomer probes of SEQ. ID. NOs. 3 to 30, wherein the oligomer probes of SEQ. ID. NOs. 3 to 20 are species-specific oligomer probes capable of binding to a specific DNA sequence of a specific mycobacterial species and wherein the oligomer probes of SEQ. ID. NOs. 21 to 30 are capable of hybridizing with specificity to mutants of the *rpoB* gene which confer drug resistance on *M. tuberculosis* or which are also capable of hybridizing to the wild type *rpoB* gene.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The present invention is explained in detail below.

In step (1), the samples are prepared from patients who are carriers or expected carriers of *M. tuberculosis*. Because the Mycobacterium infects the lungs, samples are preferably obtained from patient expectoration. The isolation of DNA from said samples may be performed by methods commonly known to a person skilled in the art.

In step (2), the primers MOTT-rpo-long-B-5' (SEQ ID NO. 1) and TR8-long-NB-3' (SEQ ID NO. 2) are used to amplify a 531bp fragment of the Mycobacterium *rpoB* gene by PCR, as illustrated in Figure 1. As shown in Figure 2, the 531bp PCR product includes i) a conserved sequence native to all Mycobacterium, ii) a species-specific polymorphic sequence, and iii) the sequence which, when mutated, confers drug

resistance upon the host *M. tuberculosis*. Said amplified PCR product of the mycobacterial *rpoB* gene may be used to identify and distinguish between *M. tuberculosis* and MOTTs, and to detect a mutation in the *rpoB* gene. Such a mutation in the *rpoB* gene renders *M. tuberculosis* resistant to drugs such as rifampin, rifamycin, or derivatives thereof.

In step (3), the *rpoB* gene PCR product obtained in step (2) is hybridized with the oligomer probes of Table 1 below using the reverse blot hybridization method (Sambrook and Russell, Molecular Cloning: A laboratory manual, third edition, Cold Spring Harbor Press, New York, 2001). The oligomer probes of Table 1 below include oligomer probes that are capable of hybridizing with a specific DNA sequence of a specific species of Mycobacterium, and that are capable of hybridizing with specificity to the wild type *rpoB* gene or to mutated DNA sequences of the *rpoB* gene which confer resistance upon the host *M. tuberculosis* to drugs such as rifampin.

Table 1.

Name of Oligomer Probes	Sequence of Oligomer probe	Mycobacterium for targeting	SEQ ID NO.
MYC	GACGTCGTCGCCACCATCGA	All types of Mycobacteria	3
MTB	CATGTCGGCGAGCCC	<i>M. tuberculosis</i> complex	4
AVI	AAACGGTGAGCCGATCACC	<i>M. avium</i>	5
INT	AAACCTGCACGCGGGCGA	<i>M. intracellulae</i>	6
SCR	AAAAACGTACGGATGGCCAGC	<i>M. scrofulaceum</i>	7
KAN-I	AAAGGCCACGATGACCGTG	<i>M. kansasii</i> type I+V	8
KAN-II	AAAAATCTCAGGATGGCCAGC	<i>M. kansasii</i> type II+III+IV	9
GAS	AAAAATCTCAGGGTGGCCAGG	<i>M. gastri</i>	10
FOR-C	CCTGAACGCCGGCCAG	<i>M. fortuitum</i> complex	11
PER	GTTCCGGTCGAGGTGG	<i>M. peregrinum</i>	12
CHE	AAATGGTGACTGCCACCACG	<i>M. chelonae</i>	13
ABS	AAAAGGTGACCACCACCACC	<i>M. abscessus</i>	14
ULC	GGCCAGCCCATCACC	<i>M. ulcerans</i>	15
GEN/SIM	CCAGCCGACGATGACG	<i>M. genavense</i> / <i>M. simiae</i>	16
GOR-I	AAAGTCGGCGATCCGATCA	<i>M. gordonae</i> type I, III, IV	17
GOR-II	AAAAACGTCGGCAAGCCGA	<i>M. gordonae</i> type II	18
SZU	AAATCTGAACGTCGGCGAG	<i>M. szulgai</i>	19
TER	AAAGCTCAGGACGGTCAGT	<i>M. terrae</i>	20

WT1	AACCAGCTGAGCCAATTC	Wild Type 509-514	21
WT2	ATGGACCAGAACAAACCCG	Wild Type 515-520	22
WT3	AAACTGTCGGGGTTGACC	Wild Type 521-525	23
WT4	TTGACCCACAAGCGCCGA	Wild Type 524-529	24
WT5	CTGTCGGCGCTGGGGC	Wild Type 530-534	25
MT1	CTGTTGGCGCTGGGGC	Mutant Type 531TTG	26
MT2	AAAACCAACAAGCGCCGA	Mutant Type 526AAC	27
MT3	AATGGTCCAGAACAACCCG	Mutant Type 516GTC	28
MT4	AAAGCTGACCCCATTTCAT	Mutant Type 513CCA	29
MT5	AAAGCCGAGCCCATTTCAT	Mutant Type 511CCG	30

The oligomer probes described by SEQ ID NOs. 3 to 20 in Table 1 were constructed to hybridize with a specific DNA sequence of a specific species of *Mycobacterium*.

The oligomer probes WT1, WT2, WT3, WT4 and WT5 (SEQ ID NOs. 21-25) can be used to identify the wild type *rpoB* gene of *M. tuberculosis* by hybridizing with nucleotide sequences 509-514, 515-520, 521-529, 525-529 and 530-534, respectively, of the PCR product. The oligomer probes MT1, MT2, MT3, MT4 and MT5 (SEQ ID NOs. 26-30) can be used to detect mutations in the *M. tuberculosis rpoB* gene, specifically, TTG mutation of nucleotide 531, AAC mutation of nucleotide 526, GTC mutation of nucleotide 516, CCA mutation of nucleotide 516, and CCG mutation of nucleotide 511, respectively.

Figure 3 shows the absence of bands in the reverse blot hybridization using the oligomer probes WT1-5. The failure of the probes to bind to their DNA recognition sequences indicates the presence of mutations in the region encoding susceptibility to rifampin. Therefore, the detection of said mutant types enables the determination of *M. tuberculosis* resistance to rifampin.

The oligomer probes in Table 1 were designed to hybridize at the same temperature and be highly specific for a particular species of *Mycobacterium*. To achieve these purposes, the length, GC content and positions of possible mismatch in the

oligomer probes were modulated. For some less sensitive oligomer probes, non-specific nucleotides were added to the 5'-terminal ends in order to increase the efficiency of hybridization with the corresponding target DNA sequence.

Stable adhesion of the oligomer probes to the membrane is a result of a covalent linkage formed between an amino group conjugated to the 5'-terminal end of the oligomer probe and a carboxyl group on the membrane surface. These covalent links prevent the oligomer probes from moving freely on the membrane during the reverse blot hybridization. The present invention employs, but is not limited to, Biodyne-C membrane (Pall Biosupport, East Hills, NY), a negatively charged nylon membrane, as any membranes with carboxyl groups on the surface may be preferably used.

In summary, the present invention provides primers for amplifying a 531bp fragment of the *M. tuberculosis rpoB* gene by PCR, the oligomer probes described in Table 1 and the membrane for adhesion to said oligomer probes, in order to specifically identify *M. tuberculosis* and MOTTs, and to detect mutations in the *rpoB* gene which confer drug resistance to the host Mycobacterium.

Further, the present invention provides a kit for the separate identification of *M. tuberculosis* and MOTTs, and for the detection of mutations in the *rpoB* gene which confers drug resistance to the Mycobacterium, using said primers, the oligomer probes of Table 1 and the membrane for adhering said oligomer probes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a genetic map of the *M. tuberculosis rpoB* gene.

Figure 2 shows the nucleotide sequence of the 531bp fragment obtained by PCR amplification of the *rpoB* gene of *M. tuberculosis*.

Figure 3 is a drawing showing results of a PCR reverse blot hybridization of the present invention used to identify *M. tuberculosis* and MOTTs, and to detect mutations in the amplified segments of the *M. tuberculosis rpoB* gene, for the determination of susceptibility to rifampin. Lanes 1-6 represent *M. tuberculosis* with a wild type *rpoB* gene and therefore retaining susceptibility to rifampin; lane 7 represents *M. tuberculosis* with a mutation in the 514-520 nucleotide region that confers resistance to rifampin; lanes 8-15 represent rifampin-resistant *M. tuberculosis* with a mutation in the 524-529 nucleotide region; lane 16 represents rifampin-resistant *M. tuberculosis* with a mutation of nucleotide 511 to CCG; lane 17 represents rifampin-resistant *M. tuberculosis* with a mutation of nucleotide 513 to CCA; lanes 18-19 represent rifampin-resistant *M. tuberculosis* with a mutation of nucleotide 516 to GTC; lane 20 represents rifampin-resistant *M. tuberculosis* with a mutation of nucleotide 526 to AAC; lane 21 represents rifampin-resistant *M. tuberculosis* with a mutation of nucleotide 531 to TGC; and lanes 22-35 represent rifampin-resistant *M. tuberculosis* with a mutation of nucleotide 531 to TTG.

Figure 4 is a drawing showing the 531bp fragment of the *rpoB* gene amplified by PCR using DNA isolated from standard mycobacterial species and the primers described by SEQ. ID. NOs. 1 and 2. The lane "M" is the Gene Ruler™ DNA size marker, lanes 1-13 show PCR products amplified from standard species of *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. szulgai*, *M. goodii*, *M. kansasii*, *M. abscessus*, *M. chelonae*, *M. gastri*, *M. fortuitum*, *M. ulcerans*, and *M. terrae*, respectively, and lane 14 is a negative control for PCR amplification.

Figure 5 is a drawing showing results of a PCR reverse blot hybridization of the present invention for standard species of *M. tuberculosis* and MOTTs, wherein PCR products amplified from each mycobacterial species are hybridized with the

corresponding species-specific oligomer probes.

Figure 6 is a drawing showing results of a PCR reverse blot hybridization of the present invention for clinically isolated MOTTs.

Figure 7 is a drawing showing results of a PCR reverse blot hybridization used to separately identify *M. tuberculosis* and MOTTs, and to detect mutations in the amplified *rpoB* gene segments of thirty-seven clinical samples of mycobacterium.

The present invention will be explained in more detail with the following experimental examples. However, the present invention should not be limited to these examples, and it should be clearly understood that many variations and/or modifications of the basic inventive concepts herein taught which may appear to those skilled in the art will still fall within the spirit and scope of the present invention.

1. Mycobacterial species

The species described in Table 2 below are used in the present experiments as standard species of *M. tuberculosis* and MOTTs. Rifampin susceptibility testing of clinically isolated *M. tuberculosis* was previously performed at the Korean Institute of Tuberculosis. Also, the identification of MOTTs was previously made using conventional microbiological and biochemical methods, such as the PCR-RFLP method developed by the present inventors.

Table 2

	Species	Strain	Source		Species	Strain	Source
1	<i>M. abscessus</i>	Pettenkofer Inst.	YUMC	23	<i>M. intermedium</i>	ATCC 51848	KIT
2	<i>M. africanum</i>	ATCC 25420	KIT	24	<i>M. kansasii</i> type I-V		Pasteur Inst.
3	<i>M. arcinogenes</i>	ATCC 35753	KIT	25	<i>M. malmoeense</i>	ATCC 29571	KIT
4	<i>M. asiaticum</i>	ATCC 25276	KIT	26	<i>M. marinum</i>	ATCC 927	KIT

5	<i>M. aurum</i>	ATCC 23366	KIT	27	<i>M. microti</i>	ATCC 19422	KIT
6	<i>M. austroafricanum</i>	ATCC 33464	KRIBB	28	<i>M. moriokaense</i>	ATCC 43059	KRIBB
7	<i>M. avium</i>	ATCC 25291	KIT	29	<i>M. mucogenicum</i>	ATCC 49650	KIT
8	<i>M. bovis</i>	ATCC 19210	KIT	30	<i>M. neoaurum</i>	ATCC 25795	KIT
9	<i>M. bovis</i> BCG	French Strain 1173P2	KIT	31	<i>M. nonchromogenicum</i>	ATCC 19530	KIT
10	<i>M. celatum</i> type I/II	ATCC 51130/ ATCC 51131	KIT	32	<i>M. parafortuitum</i>	ATCC 19686	KIT
11	<i>M. chelonae</i>	ATCC 35749	KIT	33	<i>M. peregrinum</i>	ATCC 14467	KIT
12	<i>M. chitae</i>	ATCC 19627	KIT	34	<i>M. phlei</i>	ATCC 11758	KIT
13	<i>M. fallax</i>	ATCC 35219	KIT	35	<i>M. pulveris</i>	ATCC 35154	KRIBB
14	<i>M. fortuitum</i> type I/II	ATCC 6841/ ATCC 49404	KIT	36	<i>M. scrofulaceum</i>	ATCC 19981	KIT
15	<i>M. gallinarum</i>	ATCC 19710	KRIBB	37	<i>M. smegmatis</i>	ATCC 19420	KIT
16	<i>M. gastri</i>	ATCC 15754	KIT	38	<i>M. szulgai</i>	ATCC 35799	KIT
17	<i>M. genavense</i>	ATCC 51233	KIT	39	<i>M. terrae</i>	ATCC 15755	KIT
18	<i>M. gilvum</i>	ATCC 43909	KIT	40	<i>M. thermoresistibile</i>	ATCC 19527	KIT
19	<i>M. gordonae</i> type I-IV	ATCC 14470	KIT	41	<i>M. triviale</i>	ATCC 23292	KIT
20	<i>M. haemophilum</i>	ATCC 29548	KIT	42	<i>M. tuberculosis</i> H37Rv	ATCC 27294	KIT
21	<i>M. intracellulare</i>	ATCC 13950	KIT	43	<i>M. ulcerans</i>	ATCC 19423	KIT
22	<i>M. interjectum</i>	ATCC 51457	KIT	44	<i>M. vaccae</i>	ATCC 15483	KIT
				45	<i>M. xenopi</i>	ATCC 19250	KIT

2. Isolation of DNA

Genomic DNA from the mycobacterial species described in Table 2 was obtained by boiling 0.4 mL mycobacterial suspensions. About 5 uL of genomic DNA was used in each PCR amplification.

For patient-derived clinical samples, DNA was isolated from patients' expectoration. The expectoration was treated with an equal volume of 4% NaOH and then the solution was homogenized by vortexing, followed by reaction at room temperature for 15 minutes. Distilled water was added to the reaction solution to a final volume of 50 mL. The solution was spun at 3,000 rpm for 20 minutes, and the supernatant was discarded. The pellet was resuspended and 500 uL of the solution was mixed with an equal volume of 2% NaOH. Next, the solution was boiled for 2 minutes and then spun at 12,000 rpm for 3 minutes, followed by discarding the supernatant. The pellet was resuspended in 1 mL of 0.1M Tris-Cl (pH 6.8), mixed well, spun at 12,000 rpm for 3 minutes, and the supernatant was discarded. Glass beads were added to the

pellet and the solution was shaken for 90 seconds. Approximately 5-10 uL of the DNA was used in the following PCR amplification.

3. PCR amplification

PCR reaction mixtures contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.001% gelatin (w/v), dNTPs (each 200 µM), *Taq* polymerase (1.25 unit), primers (10 pmol) and genomic DNA (50-100 ng) at a final volume of 50 uL. The PCR reaction was performed under the following conditions: denaturation for 5 minutes at 95°C; 35 cycles of denaturation at 94°C for 30 sec, followed by annealing at 58°C for 30 sec, followed by extension at 72°C for 45 sec; and finally, 10 minutes of extension at 72°C.

The sequences of the primers employed in the PCR reactions are as follows:

MOTT-rpo-long-B-5' : 5'-TCAAGGAGAAGCGCTACGACCTGGC-3' (SEQ ID NO.1)

TR8-long-NB-3' : 5'-ACGGGTGCACGTCGCGGACCTCCA-3' (SEQ ID NO.2)

and the region of the *rpoB* gene amplified by this PCR reaction is shown in Figure 1.

Further, the sequence of the 531bp PCR fragment is shown in Figure 2.

Confirmation of the 531bp PCR product was obtained by electrophoresis of about 5 uL of the reaction solution on a 1.0% agarose gel.

4. Oligomer probes

The oligomer probes used to separately identify *M. tuberculosis* and specific MOTTs, and the oligomer probes used to detect mutations in the DNA sequence which encode rifampin susceptibility in *M. tuberculosis*, were designed using the *rpoB* gene sequence of each specific Mycobacterium species, respectively. Each oligomer probe was capable of hybridizing with its target DNA sequence at the same temperature. Also, the sequence length and GC content of each oligomer probe was adjusted to increase the

specificity and sensitivity to its target DNA sequence. In particular, for oligomer probes which exhibited lower sensitivity to their target sequences, non-specific nucleotides were added to the 5'-terminal end in order to increase the efficiency of binding of oligomer probe to the target DNA sequence. The DNA sequences of said oligomer probes are presented in Table 1.

Stable adhesion of the oligomer probes to the Biodyne-C membranes was facilitated by the formation of covalent bonds between amino groups conjugated to the 5'-terminal ends of the oligomer probes and carboxyl groups on the surface of the membranes.

5. PCR-reverse blot hybridization

After PCR amplification, the 531bp PCR product was confirmed on an agarose gel. PCR-reverse blot hybridization was performed using 10 uL of the confirmed PCR product and oligomer probes adhered to a Biodyne-C membrane as prepared above. The oligomer probes and PCR product were loaded using a Miniblotter-MN45 (Immuntics, Cambridge, MA).

Briefly, PCR-reverse blot hybridization was performed as follows. The confirmed PCR product (10 uL) was diluted by adding 150 uL of 2x SSPE/0.1% SDS solution. Next, the diluted PCR product was denatured at 99°C for 10 minutes, followed by cooling in ice. Before loading the PCR product on the membrane, the Biodyne-C membrane was soaked in 100 mL of 2x SSPE/0.1% SDS solution at room temperature for 5 minutes and then positioned on the support cushion in the Miniblotter. After aspirating the remaining moisture in the slot, the diluted PCR product was loaded into the slot, with the slot vertically positioned in the direction of the adhered oligomer probes. The empty slot next to the slot loaded with PCR product was filled with 2x

SSPE/0.1% SDS in order to prevent cross-flow. The hybridization reaction was performed in a 50°C flat-ground incubator for 2 hours. For preventing cross-flows between neighboring slots, the membrane was not shaken.

Following hybridization, the sample was removed from the Miniblotter using an aspirator. The membrane was removed from the Miniblotter and washed two times with 100 mL of 2xSSPE/ 0.5% SDS solution at 57°C for 10 minutes. Then, the membrane was put in a rolling bottle, and 10 mL of streptavidine-alkaline phosphatase conjugate solution diluted 1:2000 with 2x SSPE/0.5% SDS solution was poured into the membrane, followed by reaction at 42°C for 60 minutes. Then, the membrane was washed two times with 100 mL of 2x SSPE/0.5% SDS solution at 42°C for 10 minutes, and two times with 100 mL of 2x SSPE solution for 5 minutes.

For chemiluminescent detection of the hybridized reaction products, the membrane was treated with 10 mL of CDP-StarTM detection reagent (Amersham Pharmacia Biotech., Buckinghamshire, England) for 4 minutes. Next, the membrane was wrapped with overhead sheet or wrap and exposed to X-ray film for 30 minutes (if needed, further exposure to X-ray film for up to 2 hours), thereby yielding the experimental results described in the following section.

5. Results

1) PCR amplification of the *rpoB* gene from standard species of *Mycobacterium*

DNA was isolated from 54 standard species of *Mycobacterium* (furnished by ATCC), including subtypes kept in the Korean Institute of Tuberculosis. The *rpoB* gene regions were amplified from the samples by PCR as described previously. As shown in Figure 4, 531bp PCR fragments, which include the DNA sequence used for separately

identifying *M. tuberculosis* and MOTTS and the DNA sequence that encodes for resistance to antituberculosis drugs, were amplified using the primers MOTT-rpo-long-B-5' and TR8-long-NB-3', irrespective of species type (*see*, Figure 1).

2) PCR-reverse blot hybridization using amplified *rpoB* gene products from standard species of *Mycobacterium*

The PCR product amplified from genomic DNA isolated from each standard species of *Mycobacterium* was hybridized specifically with the oligomer probe that was designed for that species, thereby confirming the separate identification of each mycobacterial species. The results are shown in Figure 5. Most of the standard mycobacterial species bound to the oligomer probe designed to target all *Mycobacterium* (SEQ ID NO. 3, Table 1), and all the species-specific oligomer probes bound to the PCR products to which they were designed to have specificity for. These results indicate that the oligomer probes according to the present invention may be used to separately identify *M. tuberculosis* and MOTTs. Moreover, the species-specific oligomer probes did not hybridize with PCR products amplified from mycobacterial species that they were not designed to have specificity for.

As exemplified by *M. gordonae*, *M. kansasii* and *M. fortuitum* in Figure 5, the same species may have different subtypes which share some sequence polymorphism. Therefore, the oligomer probes of the present invention were designed to hybridize with as many subtypes of the same species as possible. Although DNA isolated from *M. terrae* did not hybridize with the oligomer probe that targets all *Mycobacterium*, it did specifically hybridize with the oligomer probe specific to *M. terrae*. Moreover, although the DNA isolated from *M. genavense* hybridized with the oligomer probe specific to *M. tuberculosis*, it also hybridized specifically with the oligomer probe specific to *M.*

genavense.

3) PCR-reverse blot hybridization using amplified *rpoB* gene products from clinical *Mycobacterium* species isolated at the Korean Institute of Tuberculosis

The identification of clinical mycobacterial species was performed using PCR-reverse blot hybridization as described previously. Clinical mycobacterial species were previously identified by microbiological, biochemical, and molecular biological methods such as PCR-RFLP. These identified mycobacterial species include *M. tuberculosis* and MOTTs with clinical significance or with high frequency of appearance. Examples of such MOTTs include pathogenic species such as *M. avium-intacellulare* complex, *M. kansasii*, *M. marinum*, *M. fortuitum*, *M. chelonae*, and *M. abscessus*; pathogenic MOTTs with less frequency of appearance include *M. malmoense*, *M. asiaticum*, *M. xenopi*, *M. simiae*, *M. scrofulaceum*, *M. nonchromogenicum*, *M. peregrinum*, *M. szulgai*, *M. haemophilum*, and *M. ulcerans*; and, non-pathogenic MOTTs with high frequency of appearance include *M. terrae* and *M. gordonae*. Among these, MOTTs that are generally available at the Korean Institute of Tuberculosis include *M. avium*, *M. intracellulare*, *M. fortuitum*, *M. terrae*, *M. gordonae*, *M. chelonae*, and *M. abscessus*.

PCR-reverse blot hybridization was performed with MOTTs species with a high frequency of appearance in Korea. As shown in Figure 6, each clinical mycobacterial species hybridized to the oligomer probe to which it had specificity for. Identification of *Mycobacterium* species using the method of the present invention confirmed the results obtained by previous microbiological, biochemical and molecular biological methods. Further, using the method of the present invention, it was confirmed that various species of MOTTs exist in mixed populations. This result was not previously obtained with microbiological and biochemical analyses, such as PCR-RFLP.

4) Identification of *M. tuberculosis* and determination of susceptibility to rifampin

In order to determine the rifampin susceptibility of an identified sample of *M. tuberculosis*, PCR-amplified segments of both the wild type and mutated *rpoB* gene were hybridized with oligomer probes which target specific mutations in the gene which confer resistance to rifampin, using the PCR-reverse blot hybridization method and oligomer probes described previously. As shown in Figure 3, PCR-amplified sequences with specific mutations that confer rifampin resistance to *M. tuberculosis* were detected and distinguished from the wild type DNA sequence using the oligomer probes and method of the present invention.

5) Identification of Mycobacterium and determination of drug susceptibility to rifampin using DNA isolated from patient expectoration samples

DNA was isolated from expectoration samples of thirty-seven patients diagnosed as smear positive by acid fast staining (Laboratory services in Tuberculosis control, Part II. Microscopy, Global Tuberculosis Programme, World Health Organization 1998). The *rpoB* gene region was amplified by PCR using the isolated DNA and the amplified PCR products were subjected to PCR-reverse blot hybridization as described previously, in order to identify the species of mycobacterium and to determine the drug susceptibility of identified *M. tuberculosis* samples to rifampin. As a result, the mycobacteria in smear positive expectoration samples were separately identified as *M. tuberculosis* or MOTTs. The mycobacteria identified as *M. tuberculosis* were subsequently determined to be either rifampin-susceptible or rifampin-resistant, based on detection of drug-resistant genotypes using *rpoB* gene mutation-specific oligomer probes. As shown in Figure 7, *M.*

tuberculosis was identified in all the samples; the sample in lane 29 in particular was determined to be rifampin-resistant *M. tuberculosis*.

In summary, the method according to the present invention may be used to separately identify *M. tuberculosis* and MOTTs and, for species identified as *M. tuberculosis*, lead to the determination of resistance to antituberculosis drugs such as rifampin by the detection of nucleotide mutations in the *rpoB* gene. The present invention reduces the time required for Mycobacterium identification and determination of drug resistance to a total of 1-2 weeks, compared to conventional methods which require a total of 3-4 weeks. Further, the method of the present invention does not expose experimenters to extensive contact with harmful mycobacteria while providing the means to obtain rapid and accurate results.

References

1. Kox LFF, van Leeuwen J, Knijper S, Jansen HM and Kolk AHJ: PCR assay based on DNA coding for 16S rRNA for detection and identification of Mycobacteria in clinical samples. *J Clin Microbiol* 33: 3225-3233, 1995.
2. Sanguinetti M, Posteraro B, Ardito F, Zanetti S, Cingolani A, Sechi L, de Luca A, Ortona L and Fadda G: Routine use of PCR-reverse cross-blot hybridization assay for rapid identification of *Mycobacterium* species growing in liquid media. *J Clin Microbiol* 36: 1530-1533, 1998.
3. Garcia, M. J. and E. Tabares : Separation of *Mycobacterium gadium* from the rapidly growing mycobacteria on the basis of DNA homology and restriction endonuclease analysis. *J. Gen. Microbiol.* 132: 2265-2269, 1986.
4. Rubina, P., J. T. Kuach, and P. Mounts: Isolation and restriction endonuclease

analysis of mycobacterial DNA. *J. Gen. Microbiol.* 132: 541-551, 1986.

5. Bai, G. H. : Rapid identification of *Mycobacterium avium* and *Mycobacterium intracellulare* by the amplification of rRNA sequences. *J. Kor. Soc. Micro.* 27(5): 443-448, 1992.

6. Lee, H., H. Park, S. Cho, G. Bai and S. Kim. 2000. Species identification of Mycobacteria by PCR-Restriction Fragment Length Polymorphism of the *rpoB* gene. *J. Clin. Microbiol.* 38:2966-2971.

7. Kamerbeek, J., Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, A. Bunschoten, H. Molhuizen, R. Shaw, M. Goyal, and J. van Embden. 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J. Clin. Microbiol.* 35:907-914.

8. Woods, G. L. and T. A. Washington II: Mycobacteria other than *Mycobacterium tuberculosis* : Review of microbiologic and clinical aspects. *Rev. Inf. Dis.* 9(2): 275-294, 1987.

9. Jenkins, P. A. : Mycobacteria in the environment. *J. Appl. Bact. Sym. Suppl.* 70: 1375-1415, 1991.

10. Tsukamura, M., H. Shimoide, A. Kuse : Epidemiologic studies of lung disease due to mycobacteria other than *Mycobacterium tuberculosis* in Japan. *Rev. Inf. Dis.* 3(5): 997-1007, 1981.

11. O'Brien, R. J., Geiter, L. J., and Snider, D. E. : The epidemiology of nontuberculous mycobacterial diseases in the United States. Results from a national survey. *Am. Rev. Respir. Dis.* 135: 1007-1014, 1987.

12. Bai, G. H., Park, K. S., and Kim, S. J. : Clinically isolated mycobacteria other than *Mycobacterium tuberculosis* from 1980 to 1990 in Korea. *J. Kor. Soc. Micro.* 28(1): 1-5, 1993.